Submitted: 13 May, 2025 Accepted: 31 July, 2025 Published: 03 January, 2026 DOI:10.22514/jocpd.2026.018

ORIGINAL RESEARCH

Journal of Clinical Pediatric Dentistry

In vitro evaluation of the bactericidal effect of human breast milk against *Streptococcus mutans* at three time periods

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Abstract

Background: Human breast milk is a dynamic and multi-faceted fluid that contains essential nutrients for infant health and development. Its composition changes throughout the stages of lactation, and besides providing the newborns with plenty of nutrients, it also protects them against infections such as sepsis, pneumonia, and enteritis, especially in premature infants. The question of whether breastfeeding is cariogenic or not remains unresolved when comparing the results of different researchers. The objective of this study was to measure the bactericidal effect of breast milk against *Streptococcus mutans* (*S. mutans*), which has a high cariogenic potential. **Methods**: Breast milk samples were collected from 9 donors and divided into 3 groups: Group 1 (fresh milk), Group 2 (milk stored at −18 °C for 24 hours) and Group 3 (milk stored at −18 °C for 72 hours). From each group, 3 disks soaked in milk were placed on an agar culture of *S. mutans*. In the same culture a disk soaked with chlorhexidine was placed as a control. Inhibition halos were measured after 24, 48 and 72 hours. **Results**: Only Group 1 (fresh milk) incubated for 24 hours showed a statistically significant difference, presenting the lowest inhibition ($p < 0.05$). In the other eight data sets, there was no significant difference showing higher or lower inhibition ($p > 0.05$). However, there was a trend toward reduced inhibitory capacity when the milk was frozen for 72 hours. **Conclusions**: Human breast milk showed a bactericidal effect against *S. mutans*, which has a high cariogenic potential. Given this, we recommend exclusive breastfeeding during the first 6 months of life and thereafter combining breastfeeding and complementary feeding for as long as desired by the child and mother.

Keywords

Human breast milk; *Streptococcus mutans*; Bactericidal effect; Cariogenic

1. Introduction

Human breast milk, besides being an essential source of nutrients for early human growth and development, contains several essential immunological components with anti-infective activities and critical functions in the maturation of the immune system. It is also known that maternal milk contains its own unique microbiome, which includes beneficial, commensal and potentially probiotic bacteria [1]. In a study by Saeed *et al*. [2] (2023), probiotics of the genera Staphylococcus and Streptococcus were identified from breast milk cultures and showed significant effects on antibiotic resistance.

Breast milk releases functional peptides that benefit both maternal and infant health by preventing bacterial infection and modulating the immune system [3]. Its oligosaccharides may act directly as antimicrobial agents by inhibiting bacterial adhesion to epithelial cells. They are also sialic acid precursors, which is in turn a fundamental part of brain gangliosides and glycoproteins. A 2017 study described a novel endogenous peptide cleaved from β-casein, named β-casein 197, which has antimicrobial activity. It was found that three bacterial strains are sensitive to the antibacterial effect of β-casein 197: *Escherichia coli*, *Streptococcus aureus* and *Y. enterocolitica* [4]. Numerous peptides derived from human breast milk through *in vitro* proteolysis perform functions beyond being a simple source of nutrients like amino acids. Breast milk peptides have additional functions such as immunomodulation, opioid-like activity, antimicrobial action, and probiotic action [3].

In contrast, the antimicrobial activity of lactobacilli is mainly related to the production of organic acids such as lactic acid, acetic acid, propionic acid and, sometimes, hydrogen peroxide, bacteriocins, and antimicrobial peptides [5]. The relation between human breast milk and the development

of dental caries has been discussed in the literature with controversial results [6, 7].

The early oral environment, primarily shaped by maternal factors, quickly evolves into a more complex and mature ecosystem influenced by external conditions. It is important to identify, at an early age, the factors that trigger oral disease later in life. For example, healthy 3-month-old infants already host potentially cariogenic bacteria (*S. mutans*) whose numbers increase with age. Conversely, other Streptococcus species, such as *Streptococcus salivarius* and *Streptococcus mitis*, are associated with pH maintenance through alkali-generating pathways, and therefore they are linked to a protective effect against caries [8]. Early childhood caries (ECC) is a chronic disease which affects children's oral health globally. It is a multifactorial disease, but the primary risk factor is the presence of cariogenic microorganisms such as *S. mutans* [9].

Although *S. mutans* is not solely responsible for the development of dental caries, it has been shown that it may modify the local environment by producing a habitat rich in extracellular polymeric substances (EPS) and reducing the pH, which favors the growth of other aciduric and acidogenic bacteria [10].

2. Materials and methods

2.1 Type and place of study

This was an experimental, analytical, and prospective study, performed at the Microbiology Laboratory of the Faculty of Dentistry and at the Biology Cell and Ultrastructure Department of the Faculty of Medicine, both part of the Autonomous University of Coahuila in Torreón. The aim was to measure the bactericidal effect of breast milk against *S. mutans*, a bacterium with high cariogenic potential.

2.2 Collection and handling of breast milk

Breast milk samples were collected from nine donors selected through non-probability convenience sampling. All participants were healthy, well-nourished, and without medication antecedents. The samples were obtained by mechanical extraction using a Philips Avent electric breast pump (SCF395/11, Amsterdam, Netherlands) and collected in a sterilized plastic bottle of the same device. All the milk of one breast was collected and transported at room temperature in 20 minutes or less to the laboratory and it was divided in three portions. All donors gave their informed consent before giving the samples.

2.3 Study population

The sample was selected for convenience, since it is a non-probabilistic sampling and does not require randomization. The sample was established and was not determined by the availability of participants, although it is not representative of the total population.

2.4 Study groups

Each breast milk sample was divided into three portions:

Group 1: Ten drops (5 μL per drop) from each sample were collected and placed in an Eppendorf tube no later than 20 minutes after breast extraction.

Group 2: samples were labeled with the corresponding sample and donor identification numbers, then stored in a freezer at −18 °C for 24 hours. This temperature was chosen since it is commonly used by lactating women who use home-freezing for keeping and storage of breast milk. The sample was then unfrozen at room temperature and then 1000 μL were collected and placed in an Eppendorf tube.

Group 3: The sample was labeled with the donor identifying numbers, and stored in a freezer at −18 °C for 72 hours. Afterward, the sample was unfrozen at room temperature and then 1000 μL were collected and transferred to an Eppendorf tube.

Mitis Salivarius Agar preparation: One liter of purified water was placed in a beaker and then 90 g of Mitis Salivarius Agar were added. The mixture was heated and stirred for 1 minute until the agar fully dissolved. It was then poured into Petri dishes and allowed to cool until solidified.

Filter paper disks preparation: Three sets of Eppendorf tubes were used: the first containing maternal milk samples, the second containing 1000 μL of saline solution as a negative control, and the third containing 1000 μL of chlorhexidine as positive control. Three 6 mm filter paper disks were added to each tube.

Inoculum preparation: An inoculum of *S. mutans* with registration code ATCC 25175 was used. A sterile cotton swab was immersed in the inoculum, until fully soaked. Excess liquid was drained over the walls of the container. The samples were swabbed on the agar in the Petri dishes to obtain a confluent growth by compact parallel streaking of the cotton swab all over the surface of the dish. This process was repeated three more times, rotating the dish 60° each time. The culture was allowed to dry for 5 min before adding the filter paper disks.

Filter paper disks placement: The filter paper disks were placed over the agar in the Petri dishes using sterile tweezers, positioned at least 15 mm away from the edge and making sure the inhibition halos would not superpose. The Petri dishes were then incubated at 37 °C in an atmosphere with 5% CO_2 and 95% air, at temperature of 37 °C for 24 hours.

Inhibition halos measurements: Three inhibition halo measurements were taken for each filter paper disk: after 24 hours, 48 hours and 72 hours of incubation (Fig. 1).

2.5 Statistical analysis

The data was analyzed to determine the distribution of the kurtosis, symmetry, and homogeneity of the variances (Brown-Forsyte). After verifying the homogeneity of the variables, analysis of variance (ANOVA) was used to determine the difference between the experimental groups using an alpha value of $p < 0.05$. The statistical package used was SPSS (version 23, IBM, Armonk, NY, USA).

3. Results

Nine experimental data sets were analyzed by one-tail ANOVA of the inhibition halos on each sample of maternal milk tested (fresh, after 48 hours frozen and after 72 hours frozen) at the three incubation times (24, 48 and 72 hours).

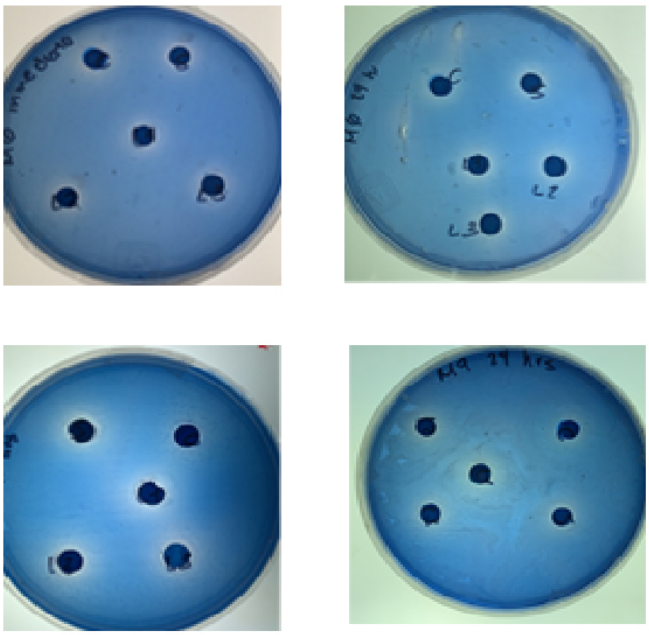

FIGURE 1. Inhibition halos measurements.

Table 1 shows the results of the incubation periods, as well as the probability value. It can be observed that in G1 with fresh milk at 72 hours of incubation, there is a trend, although not statistically significant ($p > 0.05$).

TABLE 1. Results of the incubation periods.

Group/Freshness/Incubation	*p* Value
G1/Fresh/24 h incubation	0.032
G1/Fresh/48 h incubation	0.106
G1/Fresh/72 h incubation	0.069
G2/Frozen 24 h/24 h incubation	0.189
G2/Frozen 24 h/48 h incubation	0.343
G2/Frozen 24 h/72 h incubation	0.325
G3/Frozen 72 h/24 h incubation	0.639
G3/Frozen 72 h/48 h incubation	0.704
G3/Frozen 72 h/72 h incubation	0.499

Group 1 (fresh milk) incubated for 24 h showed statistical difference ($p < 0.032$), being the lowest of the inhibitions. In the other eight data sets, there was no statistical difference showing higher or lower inhibition (Table 1).

Dunnett's multiple comparison test was used to identify significant differences between the experimental groups and a control group.

Table 2 shows the averages in the control group versus Group 1, which corresponds to the fresh milk group, for the different types of incubation. There was a significant difference ($p < 0.05$) in the reading after 24 h incubation when comparing against chlorhexidine inhibition.

Table 3 shows the results between the control group and Group 2, which represents milk frozen at 24 hours. No significant differences ($p < 0.05$) were found in any readings when compared to chlorhexidine inhibition.

Table 4 shows the results of the comparison between the control group and group 3, which represents frozen milk at 72 hours. No significant differences ($p < 0.05$) were found in any readings when comparing against chlorhexidine inhibition.

When comparing all groups, no statistical difference was found, however, Fig. 2 shows that there is a trend in the reduction of inhibition capacity if the milk is frozen 72 h.

TABLE 2. Group 1 (Fresh milk) Dunnett's multiple comparison test.

Group 1: Fresh milk	MEAN	Control	*p* Value
24 h incubation/milk1	11.444	11.778	
24 h incubation/milk2	10.000		
24 h incubation/milk3	10.444		
48 h incubation/milk1	11.000	11.556	
48 h incubation/milk2	10.111		0.032
48 h incubation/milk3	10.222		
72 h incubation/milk1	11.556	11.444	
72 h incubation/milk2	10.111		
72 h incubation/milk3	10.333		

TABLE 3. Group 2 (Frozen 24 h) Dunnett's multiple comparison test.

Group 2: Milk frozen 24 h	MEAN	Control	*p* Value
24 h incubation/milk1	11.000	11.000	
24 h incubation/milk2	9.778		
24 h incubation/milk3	10.000		
48 h incubation/milk1	10.889	10.778	
48 h incubation/milk2	9.778		0.189
48 h incubation/milk3	10.111		
72 h incubation/milk1	11.000	10.333	
72 h incubation/milk2	9.778		
72 h incubation/milk3	10.222		

TABLE 4. Group 3 (Frozen 72 h) Dunnett's multiple comparison test.

Group 3: Milk frozen 72 h	MEAN	Control	*p* Value
24 h incubation/milk1	10.333	9.11	
24 h incubation/milk2	9.778		
24 h incubation/milk3	9.556		
48 h incubation/milk1	10.111	9.00	
48 h incubation/milk2	9.889		0.069
48 h incubation/milk3	9.444		
72 h incubation/milk1	10.556	9.11	
72 h incubation/milk2	9.889		
72 h incubation/milk3	9.556		

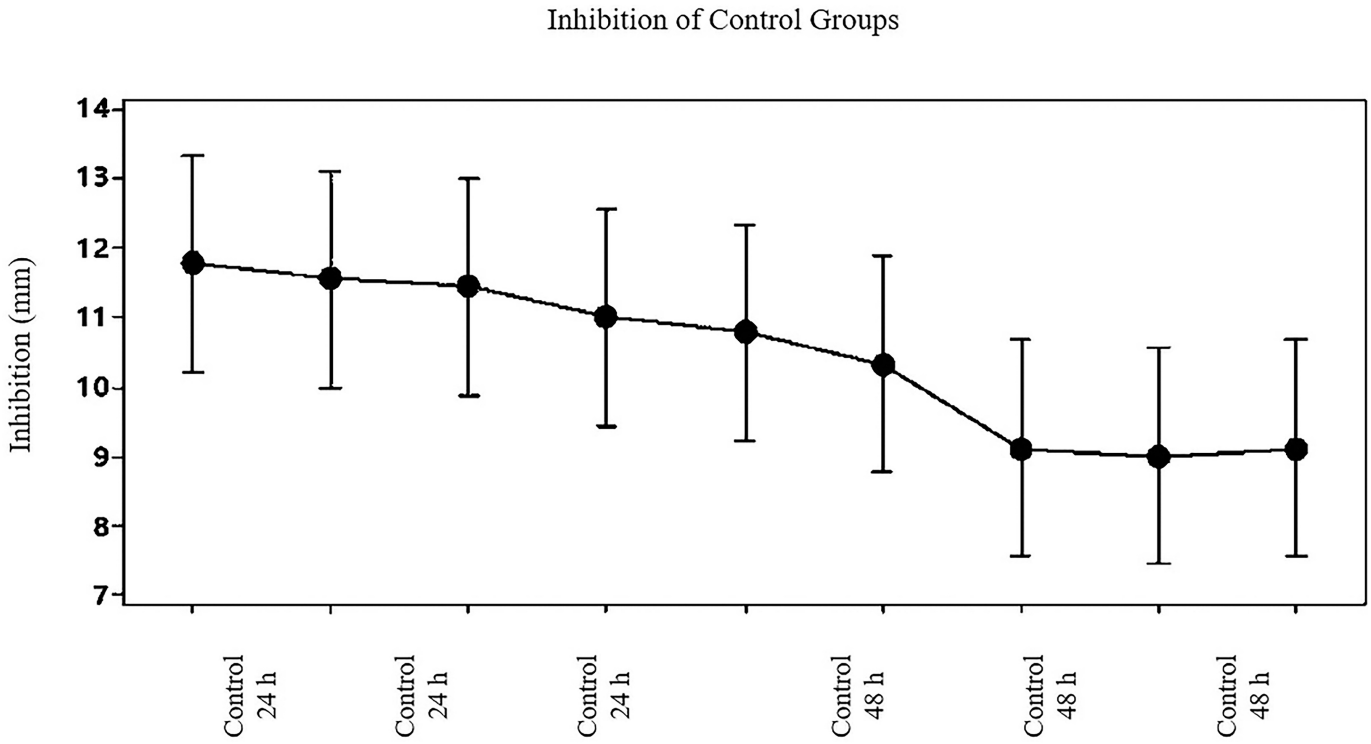

FIGURE 2. Inhibition graph of control groups.

4. Discussion

The controversy regarding the possible association between breastfeeding and dental caries remains unresolved. There has always been a disagreement in the findings of different groups regarding the correlation between breastfeeding and caries [11]. A study conducted by Łubiech K *et al.* [12] in 2020 mentions that breast milk is an important source of probiotics, similar to our study where we found sensitivity of breast milk to pathogens such as *S. mutans*.

A suppression effect on cariogenic *S. mutans* was observed in lactobacilli isolated from the oral cavity of children fed with human breast milk, but not in those fed with formula milk, which implies potential benefits of maternal milk on the oral ecosystem [13]. The American Academy of Pediatrics recommends breastfeeding for 6 months or longer, as mutually desired by the mother and the infant, [14] while the World Health Organization (WHO) recommends breastfeeding for at least 24 months. One study suggests that breastfeeding beyond certain time may increase the risk of dental caries for the infant, while another one could not confirm this association. Results often vary according to the duration of breastfeeding [15].

Kato *et al.* [16] found an association between breastfeeding for over 24 months and an increased risk of caries during early childhood, coinciding with the recommendations of the American Academy of Pediatrics and the Japan Pediatric Association [17], as well as with the report of Chaffee *et al.* [18], who concluded that prolonged breastfeeding (>12 months) is associated with dental caries and the number of teeth affected by caries [19, 20].

In contrast with these results Gomersall *et al.* [21] reported that exclusive breastfeeding for 6–11 months was significantly associated with a lower decayed, missing and filled surfaces index, and with a lower caries prevalence. Furthermore, they did not find any significant association between duration of breastfeeding and dental caries. They concluded that exclusive breastfeeding for 6–11 months may protect against dental caries on primary teeth. Prolonged breastfeeding was not associated with dental caries in that population. Gomersall *et al.* [21] discarded an association between breastfeeding duration and childhood caries, concluding that the remaining evidence is of low to very-low certainty and is insufficient for determining which, if any, other intervention types and features may be effective for preventing ECC, and in which settings [22].

Human breast milk contains a variety of substances, including host defense components, such as immunoglobulins (mainly IgA), complement system proteins, non-specific immunoglobulins, lactoferrin, lysozyme, leucocytes, and cellular decomposition products. These substances protect the infant against bacterial and viral infections during the immunodeficient period of early life, thus acting as defense mechanisms. The available scientific evidence shows that breastfeeding is more effective than bottle feeding in preventing dental caries in early childhood [19].

Over several decades, accumulated evidence has clearly shown that *S. mutans* is an important factor in dental caries development due to its capability to produce changes in the plaque's microbiome through the production of extracellular polymers and organic acids. Therefore, continuous efforts to explain how *S. mutans* detects and responds to environmental signals through interconnected circuits that govern stress tolerance and biofilm formation may facilitate the identification of new targets for caries treatment and prevention [10].

Our results showed that breastmilk produces inhibition halos against *S. mutans*. This is in agreement with the findings

by Salli *et al*. [23] who reported that 2′-fucosyllactose and galacto-oligosaccharides, which are the third most abundant component of maternal milk, reduce the adhesion of *S. mutans* to saliva-coated hydroxyapatite. In doing this, they would retard early colonization of this bacteria and reduce the future caries risk in children.

A large amount of the current recommendations regarding the preservation of human breast milk are based on microbiological and immunological considerations. Currently, breast milk conservation recommendations in neonatal units and at home indicate freezing at −18 °C when children are up to 12 months. If freezing is used to conserve maternal milk, it is very important not to damage it during unfreezing. The use of microwave ovens is not recommended for unfreezing since it may significantly reduce the immunological properties of milk (IgA's amount decreases up to 98% and lysozyme's up to 96%).

Temperature recommendations based on milk's microbiological state are room temperature from 4 to 8 hours, refrigeration (4–6 °C) from 24 h to a maximum of 48–72 h, and freezing at −18 °C for 15–90 days [24].

Other associated factors such as periodontitis, should be taken into account by the stages of development in the human population for a better understanding of oral health [25], as well as the evaluation of habits and customs such as diet, cigarette consumption, among others, in the regions of the world [26].

Human breast milk components are still being identified. More standardized studies of the composition of maternal milk are needed in order to create a comprehensive and rigorous reference that includes nutrients and bioactive factors. However, the understanding of maternal milk's composition is increasing, bringing us a better understanding of the role of maternal milk in children's health and development.

One of the limitations of our study was the number of samples, as well as the use of a reference strain of *S. mutans* that does not have the virulence characteristics of a clinical strain. Thus, we recommend conducting future studies using a clinical strain.

5. Conclusions

Human breast milk showed a bactericidal effect against *S. mutans*, which has a high cariogenic potential. Given this and considering the numerous benefits of breastfeeding, we support its use as the exclusive feeding method during the first 6 months of life and thereafter combining breastfeeding and complementary feeding as long as desired by the child and the mother.

Further studies are required to clarify the potential anticariogenic properties of human breast milk and to assess the possible risks of prolonged breastfeeding in relation to dental caries development. It is suggested to have a follow-up of more regorous studies that strengthen the findings, especially with larger sample sizes, clinical applications, and improved participant control. These are recommended to validate and expand upon these findings.

AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in this published article.

AUTHOR CONTRIBUTIONS

CFM and JAFU—oversight and leadership responsibility for the research activity planning and execution. JAFU—investigation, resources, writing-review & editing. PIGL—analysed the data, statistics and the analysis of results. MSNC and NDBM—laboratory work, methodology. SCV—collected the data, writing-review & editing. JMM—provision of study materials, reagents, laboratory samples, instrumentation. All authors reviewed the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

In our study, informed consent was obtained from all participants, who were informed about the study's objectives, potential benefits, and risks. The research protocol was approved by the Bioethics Committee of the School of Medicine at the Autonomous University of Durango, Campus Gómez Palacio (Approval number: Reference 1298/24). The Bioethics Committee is registered with the Comisión Federal para la Protección contra Riesgos Sanitarios (COFEPRIS) under registration number 221001536x0261.

ACKNOWLEDGMENT

We thank Enrique Diaz Palomares Dean of School of Dentistry of the Universidad Autónoma de Coahuila for the support for the realization of this study.

FUNDING

This research received no external funding.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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How to cite this article: Celic Farías Maldonado, José Alfredo Facio Umaña, Pedro IV González-Luna, Steffany Chamut Villarreal, Maria Soñadora Niño Castañeda, Nadia Denys Betancourt Martínez, *et al. In vitro* evaluation of the bactericidal effect of human breast milk against *Streptococcus mutans* at three time periods. Journal of Clinical Pediatric Dentistry. 2026; 50(1): 190-195. doi: 10.22514/jocpd.2026.018.